

Crystallization of cyclase-associated protein from
Dictyostelium discoideum

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Cyclase-associated protein (CAP) is a conserved two-domain protein that helps to activate the catalytic activity of adenylyl cyclase in the cyclase-bound state through interaction with Ras, which binds to the cyclase in a different region. With its other domain, CAP can bind monomeric actin and therefore also carries a cytoskeletal function. The protein is thus involved in Ras/cAMP-dependent signal transduction and most likely serves as an adapter protein translocating the adenylyl cyclase complex to the actin cytoskeleton. Crystals belonging to the orthorhombic space group *C222*, with unit-cell parameters $a = 71.2$, $b = 75.1$, $c = 162.9$ Å, have been obtained from *Dictyostelium discoideum* CAP carrying a C-terminal His tag. A complete native data set extending to 2.2 Å resolution was collected from a single crystal using an in-house X-ray system. The asymmetric unit contains one molecule of CAP.

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1. Introduction

Cyclase-associated protein (CAP) was first identified in *Saccharomyces cerevisiae* as a 70 kDa protein that co-purified with adenylyl cyclase (Field *et al.*, 1988). The gene was also found by two groups independently in genetic screens aiming at genes required for Ras signaling (Field, Vojtek *et al.*, 1990; Fedor-Chaikin *et al.*, 1990). To date, CAP homologs and isoforms have been identified in mammalian and non-vertebrate organisms (see Table 1); for a recent review, see Hubberstey & Mottillo (2002).

The widely conserved Ras proto-oncogene is one of the most frequently activated genes in tumors. Ras signals can be classified into two categories: signals controlling transcription through the mitogen-activated protein kinase (MAP kinase) cascades and signals that control cell morphology and the actin cytoskeleton *via* alternate pathways (Marshall, 1995). Adenylyl cyclase was the first protein to be identified as an interaction partner of a Ras ortholog (Toda *et al.*, 1985; Gibbs & Marshall, 1989; Broach, 1991) and is regulated by Ras-dependent activation of its enzymatic activity, the catalysis of ATP into the second messenger cAMP (Toda *et al.*, 1985; Broek *et al.*, 1985). The catalytic subunit of yeast adenylyl cyclase is constituted of five regions, which include a Ras-binding region (Field, Xu *et al.*, 1990; Minato *et al.*, 1994) and a binding region for CAP (or Srv2) (Field, Vojtek *et al.*, 1990; Fedor-Chaikin *et al.*, 1990; Mintzer & Field, 1994; Nishida *et al.*, 1998). The catalytic activity of the cyclase is activated by Ras binding, which in turn is facilitated by CAP, which probably harbors the

C-terminal farnesyl group of Ras (Shima *et al.*, 1997).

Topologically, CAP is constituted of an N-terminal domain, which is required for Ras response, a C-terminal domain carrying the cytoskeletal function, and an intermediate section harboring two proline-rich regions. With invertebrates, it has been shown both genetically and biochemically that the N-terminal region of CAP binds to the C-terminal domain of adenylyl cyclase, presumably by coiled-coil interactions (Gerst *et al.*, 1991; Mintzer & Field, 1994; Nishida *et al.*, 1998). The adenylyl cyclase binding site is conserved in mammalian CAP homologs, although they do not associate with the cyclase, most likely owing to the lack of a CAP-binding domain in mammalian adenylyl cyclases (Yu *et al.*, 1999).

The C-terminal domain of CAP binds to actin monomers *in vitro* (Freeman *et al.*, 1995). Moreover, all CAP homologs that have been studied so far have been shown to bind actin through the C-terminal region (Gottwald *et al.*, 1996; Hubberstey *et al.*, 1996; Zelicof *et al.*, 1996). Thus, it seems very likely that the cytoskeletal function of this domain of CAP is a true *in vivo* function, since CAP deletion mutants in yeast exhibit abnormal morphology and, especially, disruption of the actin cytoskeleton. This is supported by the finding that overexpression of the C-terminal domain of CAP in these deletion mutants partially restores the phenotype (Gerst *et al.*, 1991; Vojtek *et al.*, 1991; Haarer *et al.*, 1993).

The N- and C-terminal domains of CAP are connected by an intermediate section which

Table 1
Comparison of CAP gene products.

Protein	Organism	GenBank accession No.	Reference	Similarity [†] with yeast CAP (M58284) (%)	Similarity [†] with human CAP (NM_006367) (%)
CAP	<i>Homo sapiens</i>	NM_006367, M98474, L12168	Matviw <i>et al.</i> (1992)	46	—
CAP2	<i>H. sapiens</i>	NM_006366	Yu <i>et al.</i> (1994)	48	73
CAP1	<i>Mus musculus</i>	NM_007598, L12367	Vojtek <i>et al.</i> (1993)	46	97
MCH1	<i>Rattus norvegicus</i>	NM_022383, L11930	Zelicof <i>et al.</i> (1993)	46	97
CAP	<i>S. cerevisiae</i>	M58284	Field <i>et al.</i> (1988)	—	46
Srv2	<i>S. cerevisiae</i>	M32663	Fedor-Chaiken <i>et al.</i> (1990)	—	—
CAP	<i>S. pombe</i>	L16577	Kawamukai <i>et al.</i> (1992)	49	48
CAP homolog	<i>Chlorohydra viridissima</i>	X79567	Fenger <i>et al.</i> (1994)	44	52
CAP	<i>D. discoideum</i>	U43027	Gottwald <i>et al.</i> (1996)	50	53
CAP	<i>Lentinus edodes</i>	AB001578	Zhou <i>et al.</i> (1998)	50	49

[†] Pairwise sequence alignments were performed with the program *BESTFIT* of the *GCG* package (Genetics Computer Group, 1996). Similarity shows the degree of homology based on similar (not necessarily identical) amino acids.

contains a proline-rich region. In the yeast protein, this domain has been further divided into the P1 and P2 regions. While the P1 region is constituted by a 14 amino-acid sequence of unknown function, the P2 region exhibits a consensus SH3-binding motif (PXXP) and is necessary to target CAP to cortical actin patches (Freeman *et al.*, 1996; Yanagihara *et al.*, 1997; Yu *et al.*, 1999). Freeman *et al.* (1996) also demonstrated that SH3 domains are bound in the CAP P2 region *in vitro*. Furthermore, actin filament binding protein 1 (Abp1p), which contains an SH3-domain, was also shown to bind to the P2 region (Lila & Drubin, 1997).

Recently, Yu *et al.* (1999) demonstrated that the adenyl cyclase binding site of CAP is required to translocate the protein to actin cortical patches, yet binding of the cyclase to CAP is not needed. This region is also necessary for CAP self-association (see also Zelicof *et al.*, 1996) and somehow regulates access to the SH3-binding motif in the intermediate region of the protein. Intriguingly, they conclude that access to the SH3-binding motif directs the subcellular translocation and that Abp1p might be the most likely candidate for the targeting protein.

Dictyostelium CAP is a phosphatidylinositol 4,5-bisphosphate (PIP₂) regulated G-actin sequestering protein, which is present in the cytosol and shows enrichment at plasma-membrane regions. The cortical translocation is mediated by the N-terminal domain. The defects of a mutant in endocytosis and cytokinesis parallel those observed in yeast. In addition, motile behavior and development are affected in the *Dictyostelium* mutant (Noegel *et al.*, 1999). CAP used in the studies presented here was subcloned from an earlier construct (Gottwald *et al.*, 1996) and fused to a C-terminal

His₆ tag. In this report, we discuss the results of its purification, crystallization and data collection.

2. Materials and methods

2.1. Protein expression and purification

CAP from *D. discoideum* was subcloned from the construct pT7-7_CAP (Gottwald *et al.*, 1996) into the pRSET_6c vector (Schoepfer, 1993) *via* *Nde*I/*Hind*III restriction sites. With the original construct used as template, a C-terminal His₆ tag was introduced by the polymerase chain reaction using Turbo *Pfu* DNA polymerase, 10% Me₂SO₄ and the following primers in the reaction mixtures: CAP1 (coding), 5'-G GAA TTC CAT ATG TCA GAA GCA ACT ATT GTT GAA-3'; CAP2 (non-coding), 5'-CCC AAG CTT CTA ATG GTG ATG GTG ATG GTG AAT ATG TGA AGT TGA-3'. Expression was carried out in *Escherichia coli* BL21(DE3). A 1 l culture of transformed cells was grown overnight at 310 K in LB medium containing 50 µg ml⁻¹ ampicillin. The overnight culture was then used to inoculate 7 l of LB medium (50 mg l⁻¹ ampicillin), which were incubated in shaker flasks at 310 K until the absorbance at 600 nm exceeded 1.0. Cell growth was continued for 4–6 h after induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. The ampicillin concentration was increased twofold at the time of induction.

After harvesting the cells, the remaining pellets were resuspended in buffer D1 (100 mM NaCl, 1.5 mM EDTA, 5 mM benzamidine chloride, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, 20 mM Tris pH 8.0) and subjected to ultrasonication. The supernatant was separated

by ultracentrifugation (35 000 rev min⁻¹, 45 min, 277 K) and applied to an equilibrated Ni²⁺-NTA column (buffer: 100 mM NaCl, 20 mM Tris pH 8.0). After extensive washing, the protein was eluted with step-wise increasing imidazole concentrations (20, 50, 100, 200 mM imidazole in 100 mM Tris pH 8.0). The fractions beginning at 100 mM imidazole contained CAP and were pooled and dialyzed overnight against 50 mM NaCl, 20 mM HEPES pH 8.0. To separate full-length CAP from auto-proteolytically generated individual domains, anion-exchange chromatography with Q-Sepharose was performed as a second step. After equilibration of the column with 20 mM HEPES pH 8.0, the dialysate was applied and the column washed with equilibration buffer. The protein was eluted by a chloride gradient of 0–1 M NaCl, 20 mM HEPES pH 8.0. CAP eluted from 160 to 400 mM NaCl; the earlier fractions also contained the individual domain(s), (N-CAP and) C-CAP in addition to the full-length protein. The later fractions contained the full-length protein only.

The protein was concentrated using Millipore (Amicon) Centricons with a 10 kDa cutoff; the use of other ultrafiltration devices proved to trigger protein aggregation and/or subsequent binding to the filtration membrane. Typical protein concentrations used were 5–6 mg ml⁻¹. The concentrated protein was used immediately for further experiments.

2.2. Protein identification

Purified protein was subjected to liquid chromatography–electrospray ionization mass spectrometry (LC-MS) on an Agilent 1100 LC-MSD instrument (Agilent, Palo Alto, CA, USA). 100 µl of the sample (50 pmol) were loaded onto a 150 × 2.1 mm ID reversed-phase Zorbax SC-C3 column equipped with a C3 guard column at a flow rate of 200 µl min⁻¹. After an initial wash with 5% acetonitrile for 25 min, the protein was eluted with a gradient from 5 to 100% acetonitrile; the aqueous phase was constituted of 5% acetic acid in water. MS data were acquired and analyzed with the *ChemStation* software.

The observed mass of 50 345 g mol⁻¹ indicates that Met1 was processed (theoretical mass: 50 475 g mol⁻¹). A second species with a mass of 21 056 g mol⁻¹ was identified in the sample, which can be explained by cleavage of CAP into its N- and C-terminal domains (N-CAP, C-CAP). The theoretical mass for C-CAP from our CAP construct is 21 057 g mol⁻¹. The isolated N-

terminal domain was not observed in the sample.

Two protein species of approximately 50 and 20 kDa were also observed with SDS-PAGE. N-terminal amino-acid analysis of both species revealed the sequences SEATIV for the 50 kDa and MKSKNFTDKS for the 20 kDa peptide, respectively. The former sequence agrees with the full-length CAP sequence where Met1 is processed. The latter sequence is that of the C-terminal domain, C-CAP, beginning at position 279.

2.3. Crystallization

Three CAP crystals were obtained using the hanging-drop vapor-diffusion method

with 6 μ l drops (3 μ l protein solution, 3 μ l reservoir solution) equilibrated against 300 μ l reservoir solution at 285 K. The crystals grew in about seven months from 1.8 M (NH₄)₂SO₄, 0.1 M Tris pH 8.4 as plates with approximate dimensions 0.3 \times 0.3 \times 0.1 mm.

2.4. X-ray data collection and analysis

X-ray data were collected in-house and at the National Synchrotron Light Source in Brookhaven (beamline X9B). In-house data with resolution extending to 2.2 \AA (Fig. 1) were collected on a MAR Research image plate mounted on a Rigaku rotating-anode X-ray generator equipped with an OSMIC mirror system. Data set CC1 was obtained from 1 $^\circ$ rotation images of a single crystal flash-frozen after short exposure to cryobuffer [1.8 M (NH₄)₂SO₄, 0.1 M Tris pH 8.4, 23% glycerol] at 100 K processed with *MOSFLM* (Leslie, 1992). Scaling, merging and truncation were performed using programs from the *CCP4* suite (Collaborative Computational Project, Number 4, 1994). The statistics are shown in Table 2. After the diffraction experiment, the crystal was thawed, dissolved in 5 mM Tris pH 8.0 and subjected to LC-MS in order to determine the protein integrity of the crystallized species. However, no interpretable mass spectra were obtained from this sample.

The diffraction pattern was indexed in the orthorhombic space group *C222*. Examination of the *00l* reflections did not allow a clear distinction between *C222* or *C222*₁. The asymmetric unit contains one molecule as supported by the Matthews V_M parameter of 2.2 $\text{\AA}^3 \text{Da}^{-1}$, with packing dense enough to ensure that no other content of the asymmetric unit is possible.

Two other crystals were mounted at beamline X9B (NSLS, Brookhaven) after soaking them in either KBr or (C₂H₅HgO)₂HPO₂ in an attempt to obtain derivative anomalous data. While the bromide soak damaged the crystal and made data collection impossible, the mercury-soaked

Table 2

Data-collection statistics.

The numbers shown refer to space group *C222*. Values in parentheses refer to the last resolution shell.

Data set	CC1
Space group	<i>C222</i> or <i>C222</i> ₁
Unit-cell parameters (\AA)	$a = 71.2$, $b = 75.1$, $c = 162.9$
Resolution (\AA)	25–2.2
No. of measurements	313526
No. of independent reflections	21835
Completeness (%)	98.8 (98.8)
Multiplicity	6.0 (5.6)
$I/\sigma(I)$	8.4 (2.5)
Matthews V_M † ($\text{\AA}^3 \text{Da}^{-1}$)	2.2
Solvent content (%)	43
$R_{\text{merge}}^\ddagger$	0.072 (0.303)

† Matthews (1968). ‡ $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$.

crystal was heavily split and displayed multiple diffraction spots for each reflection.

2.5. Patterson search attempts

Very recently, a crystal structure of the C-terminal domain of CAP from *S. cerevisiae* was deposited with the PDB (PDB code 1kq5; A. A. Fedorov, T. Dodatko, D. A. Roswarski & S. C. Almo, in preparation). Using this complete structure and a truncated poly-Ala model thereof as search models, we tried to apply the molecular-replacement method in order to solve the structure of CAP, while being well aware of the fact that the model might not be sufficient to obtain a solution with significant correlation. Patterson search calculations with *AMoRe* (Navaza, 1994) and *COMO* (Jogl *et al.*, 2001) remained unsuccessful. Thus, we are concentrating on attempts to obtain further crystals of CAP in order to enable the search for heavy-atom derivatives.

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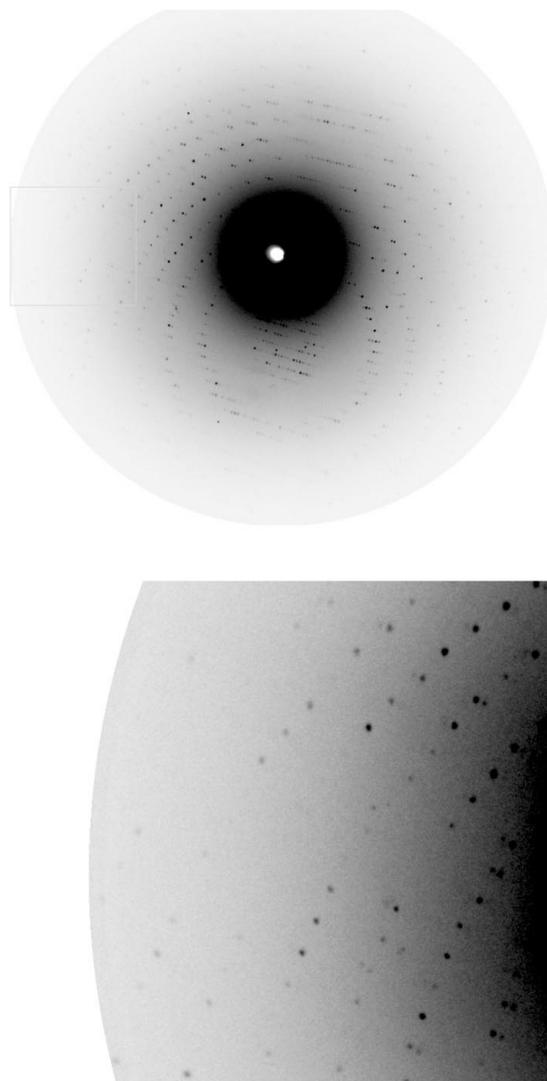


Figure 1
X-ray diffraction pattern from a crystal of *D. discoideum* CAP. The resolution at the outer edge of the image is 2.21 \AA . The upper panel shows a complete 1 $^\circ$ oscillation frame, while the lower panel shows a magnification of the area indicated (gray box).

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